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[γ-^{32}P]ATP as a tracer of the fragmentation of Ca–F-actin

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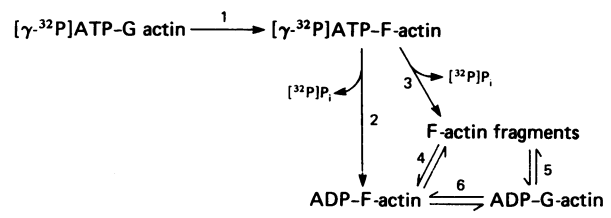
[γ-^{32}P]ATP–G-actin was polymerized in 4 mM-$CaCl_2$, and the distribution of the radioactive nucleotide among the oligomeric and the polymeric species was studied. The results obtained are best explained by assuming spontaneous fragmentation.

INTRODUCTION

Wegner (1982) and Wegner & Savko (1982) studied actin polymerization by means of light-scattering and proposed that the polymer undergoes fragmentation in the presence of either Ca^{2+} or Mg^{2+}, but not of K^+. We have recently pointed out the weaknesses of the methodology as well as of the kinetic analysis of these authors and have provided direct evidence that actin polymerized by Ca^{2+} undergoes fragmentation at 4 °C (Grazi & Trombetta, 1985).

We add here further, independent, proof that the same phenomenon occurs also at higher temperatures. This was done by studying the distribution of the actin-bound nucleotide among the oligomeric and the polymeric species formed by polymerizing [γ-^{32}P]ATP–G-actin in 4 mM-$CaCl_2$.

In this system the exchange of the nucleotide bound to actin with the nucleotide in the medium is exceedingly slow (Kuehl & Gergely, 1969). Consequently the nucleotide becomes an excellent label for actin and offers an additional signal to trace the intermediate stages of the polymerization. The results we have obtained show that [γ-^{32}P]ATP–G-actin polymerizes rapidly into long strings of [γ-^{32}P]ATP–F-actin (reaction 1 of the scheme below) which then undergo fragmentation into shorter strings of ADP–F-actin (reaction 3 of the scheme).



MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as described by Spudich & Watts (1971). Two centrifugations of F-actin at 66000 *g* were routinely included. Actin was kept at a concentration of 5 mg/ml in 0.2 mM-ATP/0.2 mM-$CaCl_2$/0.5 mM-2-mercaptoethanol/2 mM-NaN_3/2 mM-Tris/HCl buffer, pH 8.2. Actin concentration was measured from its A_{290}, the absorbance of 1 mg of pure actin/ml (light path 1 cm) being taken to be 0.62 (Gordon *et al.*, 1976). Suitable blanks for the eventual faint opalescence of the solution were obtained by measuring A_{320} and A_{360} and extrapolating back to 290 nm. Alternatively, the Coomassie Blue method was used (Bradford, 1976). The molar concentration of G-actin was calculated on the basis of an M_r of 42000 (Collins & Elzinga, 1975). Viscosity was measured with Ostwald viscosimeters (water flow time 60 s at 20 °C) maintained at 13 °C.

Carrier-free [γ-^{32}P]ATP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, as the disodium salt, was supplied from Boehringer, Mannheim, Germany.

Before each experiment G-actin (1 mg/ml) dissolved in a solution containing 40 μM-ATP, 40 μM-$CaCl_2$, 0.4 mM-NaN_3 and 2 mM-Tris/HCl buffer, pH 8.2, was incubated overnight at 2 °C in the presence of carrier-free [γ-^{32}P]ATP. Free nucleotide was then removed by treatment with Dowex 1X8 resin and polymerization was started immediately at 13 °C by diluting the actin solution with a suitable volume of a Tris buffer/$CaCl_2$ solution to yield the final concentrations of 9 μM-actin, 10 mM-Tris/HCl buffer and 4 mM-$CaCl_2$ (pH 8.2).

To determine the amount of nucleotide bound to actin, the protein solution was filtered through a Dowex 1X8 column to remove free nucleotide. After filtration, protein was precipitated by the addition of 3% (w/v) $HClO_4$ and the amount of the nucleotide released was determined at 260 nm. Bound nucleotide was found to be 95–100% of the expected value.

To make sure that actin was effectively labelled with [γ-^{32}P]ATP, 2.5 mM-$MgCl_2$ was added to a portion of the same Dowex 1 column filtrate. After 30 min of incubation at 20 °C, the polymerized actin was precipitated and the supernatant solution was extracted with butan-2-ol/benzene (1:1, v/v) (Pollard & Korn, 1973). Essentially all the radioactivity was found in the organic phase, which contained the acid phosphomolybdate complex. This shows that G-actin was labelled with [γ-^{32}P]ATP and that the radioactive phosphate was specifically released in the course of the polymerization.

P_i was determined by the method of Tashima & Yoshimura (1975) after precipitation of the protein with 0.15 M-trichloroacetic acid.

Protein sedimentation was performed on 0.2 ml samples in the TLA-100 rotor of the Beckman TL-100 centrifuge. The pellets were dissolved in 0.2 ml of 10 mM-Tris/HCl buffer, pH 8.2. Protein content was then determined by the Coomassie Blue method (Bradford, 1976). Radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter in 10 ml of a Packard emulsifier scintillation cocktail.

To make sure that the radioactive compound sedimenting with the actin pellets was [γ-^{32}P]ATP, the following experiment was performed. [γ-^{32}P]ATP–G-actin (9 μM, 2 ml) was incubated at 13 °C in the presence

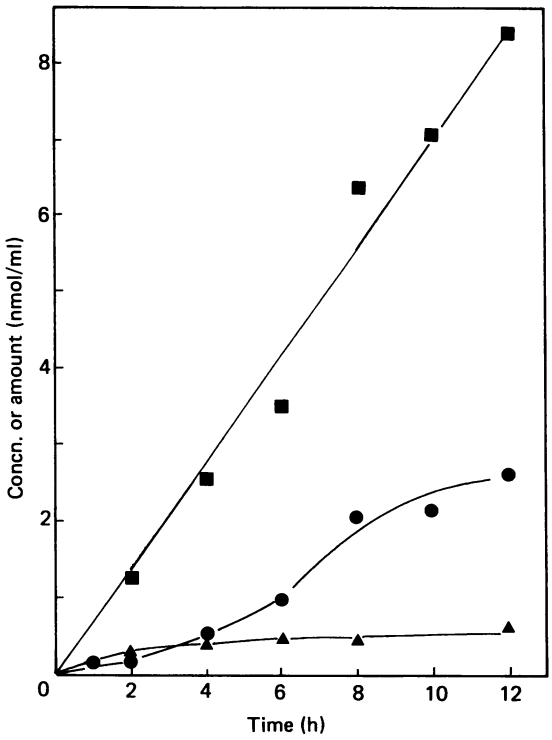


Fig. 1. Polymerization of 9 μM-[γ-^{32}P]ATP–G-actin at 13 °C and pH 8.2 in the presence of 4 mM-$CaCl_2$

●, Actin sedimented after 2 min of centrifugation at 366000 *g*; ▲, actin-bound [γ-^{32}P]ATP in the same 2 min pellets; ■, P_i released. The values on the ordinate axis refer to a concentration (orthophosphate) or to the amount of material sedimented from 1 ml of the incubation mixtures (actin, actin-bound ATP).

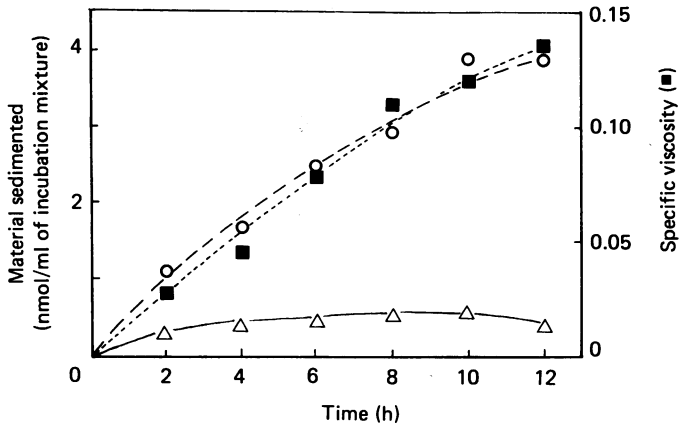


Fig. 2. Polymerization of 9 μM-[γ-^{32}P]ATP–G-actin at 13 °C and pH 8.2 in the presence of 4 mM-$CaCl_2$

○, Actin sedimented after 5 min of centrifugation at 366000 *g*; △, actin-bound [γ-^{32}P]ATP in the same 5 min pellets; ■, specific viscosity. The data in this Figure and Fig. 1 were obtained in the same experiment.

of 4 mM-$CaCl_2$. After 8 h the solution was sedimented for 2 min at 95000 rev./min (366000 *g*) and the pellets were dissolved in the original volume as previously described. Protein was precipitated by the addition of 3% $HClO_4$. The solution was neutralized with KOH and the $KClO_4$ precipitate was removed. At this stage radioactivity was in the supernatant solution. The solution was diluted to 4 ml with water and 20 mg of charcoal were added. Radioactivity was adsorbed by charcoal. This shows that the radioactive material in the pellet was ATP and not P_i.

RESULTS AND DISCUSSION

[γ-^{32}P]ATP–G-actin (9 μM; sp. radioactivity 950 d.p.m./nmol of protein) was polymerized in the presence of 4 mM-$CaCl_2$ and 10 mM-Tris/HCl, free ATP having been removed by treatment with Dowex 1X8 resin. The course of the reaction was followed by measuring (Figs. 1 and 2) (*a*) the specific viscosity of the solution, (*b*) the amount of protein sedimented after 2 min and 5 min of centrifugation at 366000 *g* in the TL100 Beckman centrifuge [we have previously shown (Grazi, 1985; Grazi & Trombetta, 1985) that, after 2 min of centrifugation, actin filaments are completely sedimented and that, after 5 min of centrifugation, the short filaments and the actin aggregates are also sedimented], (*c*) the amount of [γ-^{32}P]ATP found in the 2 min and in the 5 min protein pellets, and (*d*) the amount of P_i released. The results of a typical experiment were as follows.

(1) The formation of [γ-^{32}P]ATP–F-actin from [γ-^{32}P]ATP–G-actin (reaction 1 of the scheme shown above) is estimated directly by measuring the amount of [γ-^{32}P]ATP sedimented with the pellets. As Figs. 1 and 2 show, this amount increases in the first 6 h and then remains approximately constant at 0.5 nmol (this value refers to the amount sedimented from 1 ml of the incubation mixture).

(2) The rate of the release of P_i is approximately constant from the very beginning (0.7 μM/h) (Fig. 1). It represents the rate of formation of the ADP-containing polymeric species from the [γ-^{32}P]ATP-containing polymeric species (reactions 2 and 3 of the scheme shown above). Between 6 and 12 h, since the concentration of [γ-^{32}P]ATP–F-actin is at a steady state, the release of P_i represents also the rate of formation of [γ-^{32}P]ATP–F-actin from [γ-^{32}P]ATP–G-actin. The rate constant for the hydrolysis of ATP bound to F-actin can thus be calculated by dividing the rate of the release of phosphate (0.7 μM/h) by the [γ-^{32}P]ATP–F-actin concentration (0.5 μM, as the monomer) and is found to be 3.9×10^{-4} s^{-1}.

(3) The progress of the polymerization (reactions 1, 2 and 3 of the scheme shown above) is indicated both by the increase of the viscosity of the solution and by the increase of the amount of protein sedimented after 5 min of centrifugation. The two traces, which have been normalized, match quite nicely (Fig. 2). The amount of polymer formed at the end of the observation (12 h) is only 4 nmol out of the 9 nmol of ATP–G-actin initially present in 1 ml of the incubation mixture. The amount of P_i released over the same period of time is, in contrast, 8.5 nmol/ml of the incubation mixture (Fig. 1). This indicates that the rate of the polymerization is higher than it would appear from the rate of either the increase of the viscosity or the increase of the amount of protein sedimented after 5 min of centrifugation. A net production of ADP–G-actin is evidently taking place (reactions 5 and 6 of the scheme shown above) concomitantly with the polymerization.

(4) So far we have described a usual polymerization reaction. Starting from ATP–G-actin, ATP–F-actin is formed (Pollard & Weeds, 1984; Grazi *et al.*, 1984), which is then converted into ADP–F-actin that, in turn, equilibrates with ADP–G-actin. The interesting observation, however, is that the 2 min protein pellets (long filaments) and the 5 min protein pellets (long plus short filaments plus small aggregates) contain the same amount of actin-bound [γ-^{32}P]ATP. This means that the radioactive nucleotide is essentially all in the long filaments (Figs. 1 and 2).

A first consequence of this observation is that the [γ-^{32}P]ATP-containing oligomers, which are the precursors of [γ-^{32}P]ATP–F-actin, must be present at very low concentrations, i.e. they elongate very rapidly into structures that are sedimented after 2 min of centrifugation.

A second consequence is that, since the reaction starts from [γ-^{32}P]ATP–G-actin and the polymerization is followed by the hydrolysis of actin-bound ATP, the radioactive polymers must be of more recent formation than the non-radioactive ones. Some of the long filaments are thus 'younger' than most of the short filaments. A reasonable explanation of this finding is that F-actin (probably as ADP–F-actin), undergoes fragmentation (reaction 3 of the scheme shown above). Thus filaments are generated that are shorter and older than the radioactive ones.

After 2 h of polymerization the 2 min pellets (obtained by centrifugation of 1 ml of the incubation mixture) contain 0.16 nmol of actin and 0.165 nmol of actin-bound [γ-^{32}P]ATP. They are, therefore, formed by [γ-^{32}P]ATP–F-actin. In the 5 min pellets, actin accounts for 1.1 nmol, an amount which compares well with the P_i released (1.18 nmol). Actin-bound [γ-^{32}P]ATP remains at 0.165 nmol. These data further confirm that [γ-^{32}P]ATP–F-actin undergoes a rapid turnover with fragmentation into shorter strings of ADP–F-actin (Figs. 1 and 2).

(5) The weight of the long filaments (2 min pellets) gradually increases from 2 to 8 h as compared with the weight of the overall polymeric forms (5 min pellets). The ratios of the protein content of the 2 min to the 5 min pellets were in fact 0.14, 0.30, 0.38, 0.69, 0.66 and 0.66 at 2, 4, 6, 8, 10 and 12 h respectively. This is an indication that the reannealing reaction (reaction 4 of the scheme shown above) is taking place.

The concept that F-actin may undergo spontaneous fragmentation was first introduced by Wegner (1982) on the basis of indirect kinetic evidence. Since spontaneous fragmentation is an interesting as well as complicating feature of the polymerizing actin system, we have attempted to provide more direct evidence of the phenomenon. We have in fact directly observed, at low temperature, fragmentation of actin filaments polymerized in 4 mM-$CaCl_2$ (Grazi & Trombetta, 1985). We provide here a further, independent, proof of fragmentation, also at a higher temperature. We do not feel able at present to assess the significance of this phenomenon in the intact cell, mainly because the microfilaments are decorated with a number of ancillary proteins which certainly modify the properties of the filament itself.

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REFERENCES

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Collins, J. H. & Elzinga, M. (1975) J. Biol. Chem. **250**, 5913–5920
Gordon, D. J., Young, Y. Z. & Korn, E. D. (1976) J. Biol. Chem. **251**, 7474–7479
Grazi, E. (1985) Biochem. Biophys. Res. Commun. **128**, 1058–1063
Grazi, E. & Trombetta, G. (1985) Biochem. J. **232**, 297–300
Grazi, E., Trombetta, G. & Magri, E. (1984) Biochem. Int. **9**, 669–674
Kuehl, W. M. & Gergely, J. (1969) J. Biol. Chem. **244**, 4720–4729
Pollard, T. D. & Korn, E. D. (1973) J. Biol. Chem. **248**, 4682–4690
Pollard, T. D. & Weeds, A. G. (1984) FEBS Lett. **170**, 94–98
Spudich, J. A. & Watts, S. (1971) J. Biol. Chem. **246**, 4866–4871
Tashima, Y. & Yoshimura, N. (1975) J. Biochem. (Tokyo) **78**, 1161–1169
Wegner, A. (1982) Nature (London) **296**, 266–267
Wegner, A. & Savko, P. (1982) Biochemistry **21**, 1909–1913

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